"Factors Regulating Cytosolic Calcium Homeostasis in Cultured Vascular Endothelial Cells Exposed To Fluid Mechanical Shear Stress"

Undergraduate Thesis

Presented in partial fulfillment for the Bachelor of Science with honors research distinction at

The Ohio State University

By

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Graduating May 10, 2015

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Abstract

Calcium (Ca^{2+}) is a ubiquitous 2nd messenger, known to regulate many cellular pathways including muscle contraction, cell proliferation, and apoptosis. Deregulation of Ca^{2+} homeostasis is thought to play a role in vascular endothelial cell (EC) dysfunction, which shifts the endothelium to a pro-inflammatory state and initiates atherosclerosis. Since these cells are constantly exposed to fluid mechanical shear stress by blood flow, it is of particular interest to study the effect that shear stress has on the intracellular Ca^{2+} levels. It has been shown that when ECS are exposed to fluid shear stress, there is an increase in cytosolic Ca^{2+} levels. The three main sources of Ca^{2+}, the endoplasmic reticulum (ER), the mitochondria, and the extracellular media, are all thought to play a role in this response. However, it is not known to what magnitude each source is responsible, nor by which pathway this response is initiated. The goal of the present study was to determine the influence each source possesses in regulating the Ca^{2+} response to shear stress, and how this occurs. To monitor the Ca^{2+} response to flow, ECs were incubated with the Ca^{2+}-sensitive probe Fluo-4 and then subjected to shear stress in the presence or absence of specific chemicals to determine the source of the Ca^{2+} response. Specifically, when mitochondrial Ca^{2+} buffering was inhibited, the oscillatory Ca^{2+} response was effectively abolished. When the extracellular Ca^{2+} was eliminated, it had very little effect on the response. It was further determined that the phospholipase C (PLC)/G-protein /inositol triphosphate receptor (IP_3R) pathway is directly involved in the response, whereas the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) plays a role in maintaining oscillations. A greater understanding of these

phenomena could lead to better drug development to prevent and/or treat endothelial dysfunction, and ultimately combat cardiovascular disease.

Acknowledgements

I would like to acknowledge the guidance and encouragement provided by my research advisor, Dr. B. Rita Alevriadou, Associate Professor of Biomedical Engineering and Internal Medicine (Cardiology); Mr. Christopher G. Scheitlin, PhD student in our lab; and other past lab members whose work laid the foundation for my own. I would finally like to acknowledge the funding by NIH and OSU (the latter in the form of Kettering Scholarship, Provost Scholarship, Undergraduate Education Summer Research Fellowship, Undergraduate Research Scholarship, and Undergraduate Honors Thesis Scholarship), and Drs. Alevriadou and Janssen for serving as members of my thesis defense committee.

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- Scheitlin CG, Nair DM, Crestanello JA, Zweier JA, Alevriadou BR. "Fluid Mechanical Forces and Endothelial Mitochondria: A Bioengineering Perspective" *Cellular and Molecular Bioengineering* Julian JA's contribution was acknowledged (Dec 2014)
- Julian JA, Scheitlin CG, Alevriadou BR. "NO and Mitochondria Regulate Calcium Homeostasis in Cultured Vascular Endothelial Cells Exposed to Fluid Mechanical Shear Stress" Presented at OSU Denman Undergraduate Research Forum (March 26th, 2014)
- Julian JA, Scheitlin CG, Alevriadou BR. "Analysis of Shear Induced Calcium Oscillations in Vascular Endothelium" Presented at the OSU Fall Undergraduate Research Forum (September 3rd, 2014)
- Julian JA, Scheitlin CG, Alevriadou BR. "Emerging Determinants In Cytosolic Calcium Homeostasis in Sheared Endothelium" Presented at the National Biomedical Engineering Conference (Sept 2014)
- Scheitlin CG, Julian JA, Alevriadou BR. Manuscript in preparation (anticipated Summer 2015)

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Chapter 1: Introduction

Many cardiovascular diseases are thought to be a result of changes in the hemodynamic forces applied by flowing blood onto the inner surface of blood vessels. Atherosclerosis, a leading cause of morbidity and mortality in the United States[7], is known to cause lesions near bifurcations in arteries[6], where oscillatory flow occurs (rather than pulsatile laminar flow that occurs in straight parts of arteries). In those atheroprone areas, it has been observed that a large number of vascular endothelial cells (ECs), the cells lining the inner surface of the blood vessel, undergo programmed cell death[6], or else called apoptosis. However, the mode to which these cells sense mechanical shear stresses is not well understood. In order to prevent and treat these diseases further, a greater understanding of the mechanotransduction pathways on a cellular level is required.

To further understand the mechanisms underlying the dysfunction of the vascular endothelium, it is necessary to investigate the activity of the cells when exposed to fluid mechanical shear stress. We, and other groups in the past, found that changes in shear stress cause an oscillatory escalation of calcium concentrations ($[Ca^{2+}]_i$) within the cytosol of cultured ECs (**Figure 1**)[1,2,14,20,24]. Ca^{2+} is a vital second messenger in multiple cellular signaling cascades, and is currently being heavily studied in a wide range of fields. One major role of Ca^{2+} is, via calmodulin, the activation of endothelial nitric oxide synthase (eNOS), the enzyme responsible for production of another important 2nd messenger, nitric oxide (NO)[1, 4,11,21]. Indeed, an increase in shear stress is known to cause an increase of NO production[11,21]. However, there are also some indications that NO and reactive oxygen species (ROS) may be regulating the $[Ca^{2+}]_i$ response of

ECs to different stimuli[1,3,4,9,10,11]. It is important to note that NO is critical in vascular health since it modulates vascular tone and, hence, can directly affect blood flow.

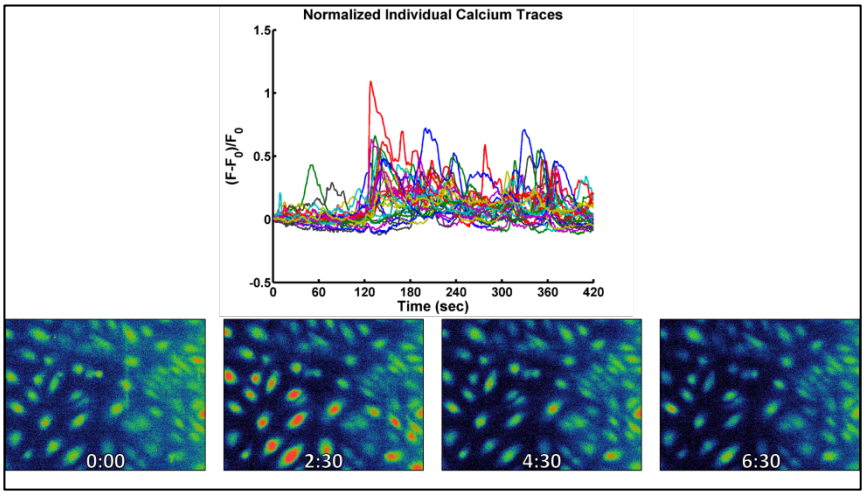

Figure 1. Shear stress causes endothelial $[Ca^{2+}]_i$ oscillations. (A) Static ECs were preincubated with Fluo-4, a calcium specific indicator, and monitored using fluorescent microscopy. Flow began at 2 min and continued for 5 min. Normalized individual cell calcium traces are shown vs. time. When flow begins, there is a large increase in $[Ca^{2+}]_i$, which is followed by oscillations. (B) Pseudo-colored images at different points in time; the different colors represent relative fluorescent intensities, blue being the lowest and red being the highest.

Another important factor in regulating the EC response to mechanical shear stress is adenosine triphosphate (ATP)[3,13,14, 17,18]. Through two modes, the P2X and P2Y receptors, ATP has been shown to elicit responses when ECs were subjected to physiological flow patterns. P2X is a direct ion channel that allows $[Ca^{2+}]$ influx through the plasma membrane (**Figure 2**)[3,14,17], whereas the P2Y channels start a cascade with phospholipase C (PLC), creating inositol trisphosphate (IP_3), and finally initiating Ca^{2+} release through the IP_3R channels in the endoplasmic reticulum (ER), the main store of Ca^{2+} within the cell[3,13,14,17,18]. There are conflicting views on whether or not P2X or P2Y play a larger role in the shear-induced $[Ca^{2+}]_i$ response.

One important, often neglected, intracellular store of Ca^{2+} is the mitochondria, which are closely localized to the ER IP_3R channels. Because of this, the mitochondria experience a microdomain of $[Ca^{2+}]_i$ much higher than the surrounding cytosol when it is released from the ER. This has led many to believe that mitochondria act as an important buffer for $[Ca^{2+}]_i$ within the cell [2,3,12,15,19,21,22,23,24], and other investigators detected a rise in mitochondrial $[Ca^{2+}]$ when a cell was exposed to a chemical stimulus[12,19,22]. When overloaded with Ca^{2+}, however, mitochondria are known to open the mitochondrial permeability transition pore (mPTP), thus releasing everything into the cytosol, including harmful chemicals that are byproducts of the electron transport chain[4,12] (**Figure 2**). This in turn initiates a form of apoptosis (the mitochondrial pathway of apoptosis), and could potentially be a factor in atherosclerosis initiation/progression.

There are many conflicting views on what causes $[Ca^{2+}]_i$ oscillations within ECs exposed to mechanical stimuli. The possible channels $[Ca^{2+}]$ traverses due to shear stress are shown in **Figure 2.** It is first necessary to understand which of these are important in the shear induced $[Ca^{2+}]$ response before exploring the complex interplay of the many other substrates involved. Once this is accomplished, further studies will be able to determine the many far-reaching effects of Ca^{2+}, and ultimately lead to better understanding of EC dysfunction/death when exposed to certain patterns of hemodynamic forces.

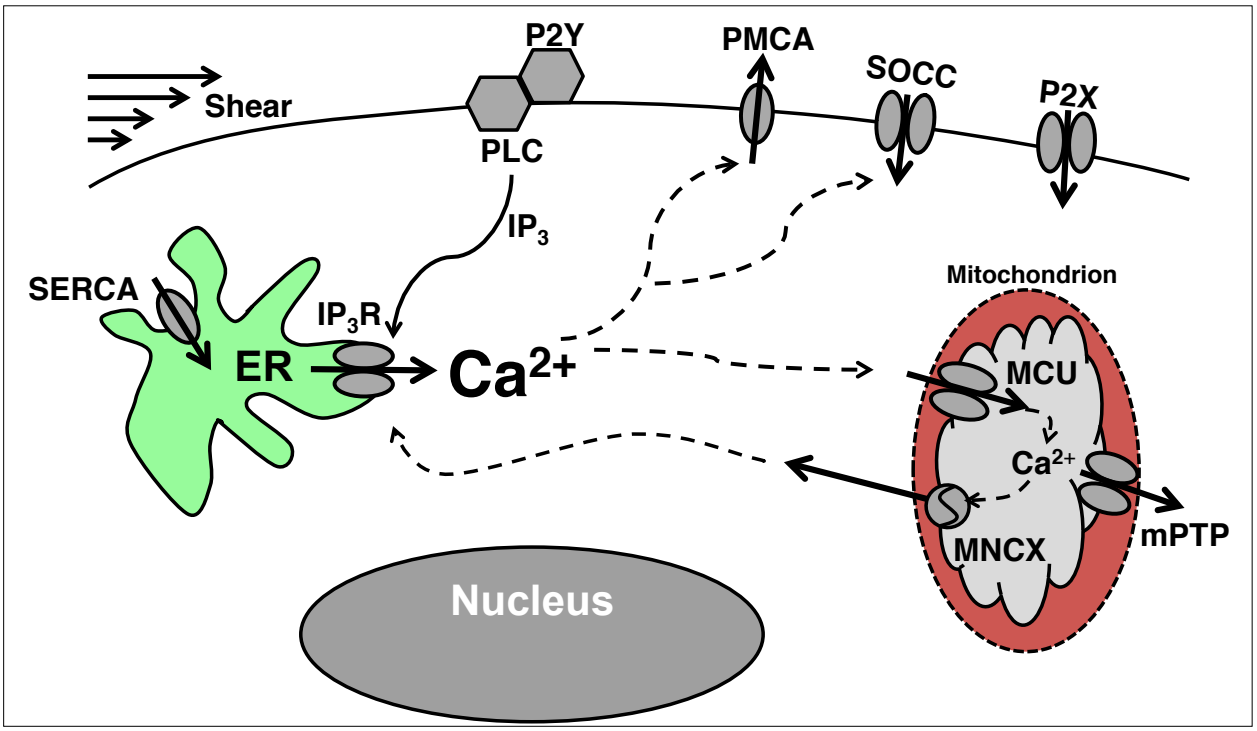

Figure 2. Schematic diagram on $[Ca^{2+}]_i$ regulation in ECs. Ca^{2+} influx through the plasma membrane (PM) occurs when extracellular ATP binds to P2X channels. During shear, binding of released ATP to P2Y receptors along the PM stimulates formation of IP_3, which binds to IP_3R on the ER and triggers Ca^{2+} release from that intracellular store. Around the same time, Ca^{2+} influx through the store-operated calcium channel (SOC) occurs. Due to the close proximity of the mitochondrial Ca^{2+} uniporter (MCU) and the IP_3R, the mitochondria have the ability to modulate $[Ca^{2+}]_i$ by uptaking Ca^{2+} in the IP_3R/MCU microdomain. Ca^{2+} is expelled from mitochondria via the mitochondrial Na^+/Ca^{2+} exchanger (MNCX). The PM and ER Ca^{2+} pumps, PMCA and SERCA, respectively, act to restore $[Ca^{2+}]_i$ to resting levels. Following mitochondrial Ca^{2+} overload, the mitochondrial permeability transition pore (mPTP) opens leading to activation of the mitochondrial pathway of apoptosis.

Chapter II. Source of Calcium Response: Extracellular

Human umbilical vein endothelial cells (HUVECs) were cultured on Ibidi μ-slide $IV^{0.4}$ parallel flow slides until confluent. These slides were connected to a gas tight syringe and a programmable syringe pump capable of producing accurate flow rates (Harvard Apparatus). During each experiment, the EC monolayers were monitored for 2 min of static incubation (0 dynes/cm^2) and then exposed to a fluid shear stress of 10 dynes/cm^2 for 5 min by perfusing Hepes-buffered saline (HBSS) buffered with 20mM HEPES over the monolayers. Changes in $[Ca^{2+}]_i$ were monitored in real time using the Ca^{2+} probe Fluo-4 AM (3 μM preincubation for 20 min) and fluorescence microscopy (Nikon) every 1 second. To examine the role of extracellular Ca^{2+}, ECs were preincubated in the normal HBSS solution and then sheared in the absence of extracellular Ca^{2+} and in the presence of 2mM lanthanum (La^{3+}). The cells were flushed gently with the $[Ca^{2+}]$ free media before being connected to the flow system. This experiment was to make sure all effects of extracellular Ca^{2+} would be eliminated, by factoring out both influx (no extracellular $[Ca^{2+}]$) and efflux (La^{3+} blocks the plasma membrane calcium ATPase). Digital fluorescence images were analyzed using ImageJ, CellProfiler, and MATLAB (**Figure 3**) in order to determine the average peak fluorescence intensity of responding cells, percent of responding cells, percent of oscillating cells, and oscillation frequency.

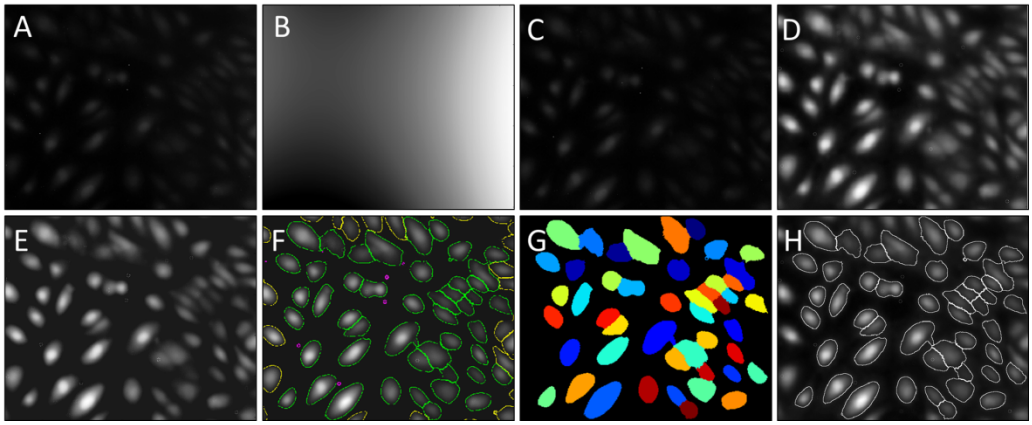

Figure. 3. Image processing and cell identification using ImageJ and the open-source software program CellProfiler. (A) A Z-stack average of the fluorescent intensities over time during the experiment. (B) Determination of uneven illumination within the field of view (C) Corrected Z-stack image. (D) Feature-enhanced image. (E) Subtracted threshold image (F) Outline identification excluding objects below size requirement or touching sides of field of view. (G) Object identification. (H) Final outlines overlaying enhanced image. These were then overlaid on each frame of the real-time fluorescence video to analyze calcium transients.

When the role of extracellular Ca^{2+} was absent, the shear response was still apparent (**Figure 4A and B**), and it was not significantly different than the controls in regards to the average peak, % oscillating, and % responding cells (**Figure 4C, D and E**). This means, in the presence of shear, the response is mostly, if not fully, due to the intracellular Ca^{2+} stores. Extracellular stores, including the store operated calcium channels (SOC), stretch activated calcium channels (SACC), and P2X channels are not involved directly with the response to shear, nor is the Ca^{2+} efflux through the plasma membrane calcium ATPase (PMCA) (**Figure 2**). However, these channels undoubtedly are responsible for maintaining Ca^{2+} basal levels and for other cellular pathways within the cell. Other extracellular components may be also vital to the response.

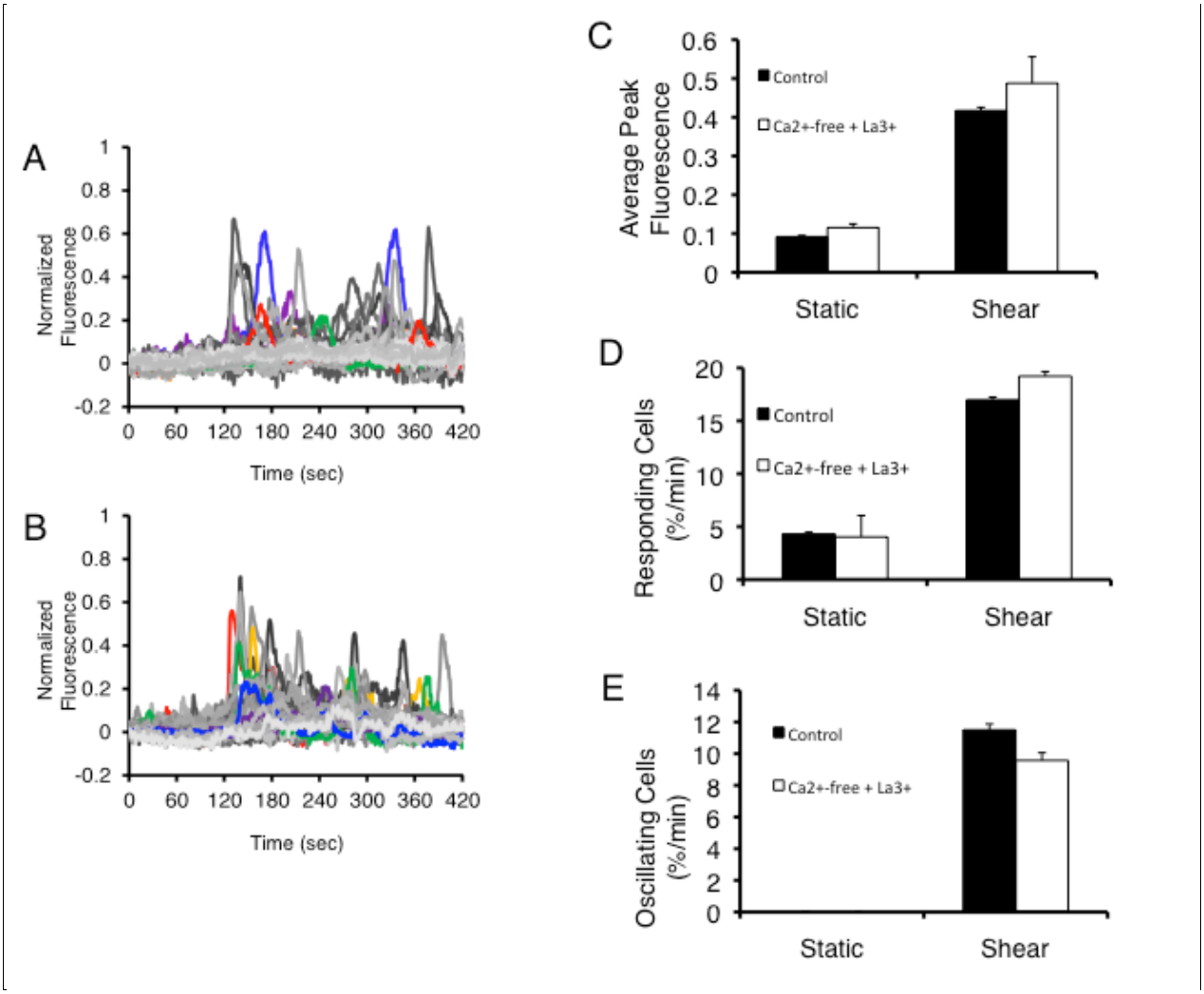

Figure 4. Effect of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ response to shear stress in ECs. A) Cells exposed to shear (10 dynes/cm^2) showed oscillatory rises in $[Ca^{2+}]_i$. B) In the absence of extracellular Ca^{2+}, cells elicited a similar response. C) Average peak response of responding cells only compared to control. D) % Oscillating cells (defined as peaking more than once) per min, to account for difference in time spans. E) % Responding cells (defined as having a significant peak) per min. * $P<0.05$ compared to static of same chemical treatment. † $P<0.05$ compared to control treatment of same mechanical treatment.

Chapter II. Source of Calcium Response: Intracellular

Experiments to determine if Ca^{2+} is released from intracellular stores, specifically the IP_3R channels of the ER, were then conducted. The methodology was the same as before, except the monolayer was now preincubated in the presence of the PLC inhibitor U73122, or its inactive analog U73343, for 60 min prior to EC connection to the flow system. U73122 is a chemical that specifically inhibits PLC, an enzyme on the plasma membrane responsible for cleaving phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3. IP_3 then binds to IP_3R on the ER to begin the release of intracellular Ca^{2+} [13,18]. Without PLC, the IP_3R pathway is effectively inhibited. The use of U73343 was to test whether the IP_3 pathway was responsible for the shear-induced $[Ca^{2+}]_i$ response.

The $[Ca^{2+}]_i$ response can be seen in **Figure 5A, B, and C.** When PLC was inhibited, the average peak fluorescence was significantly reduced compared to the control, but a response was still observed (**Figure 5D**). Interestingly, the % oscillating cells under shear in the presence of U73122 was reduced greatly compared to the control (**Figure 5E**). However, U73122 had no effect on the % responding cells compared to the control (**Figure 5F**). The inactive isotope U73343 had no measurable effect on any of the three parameters considered.

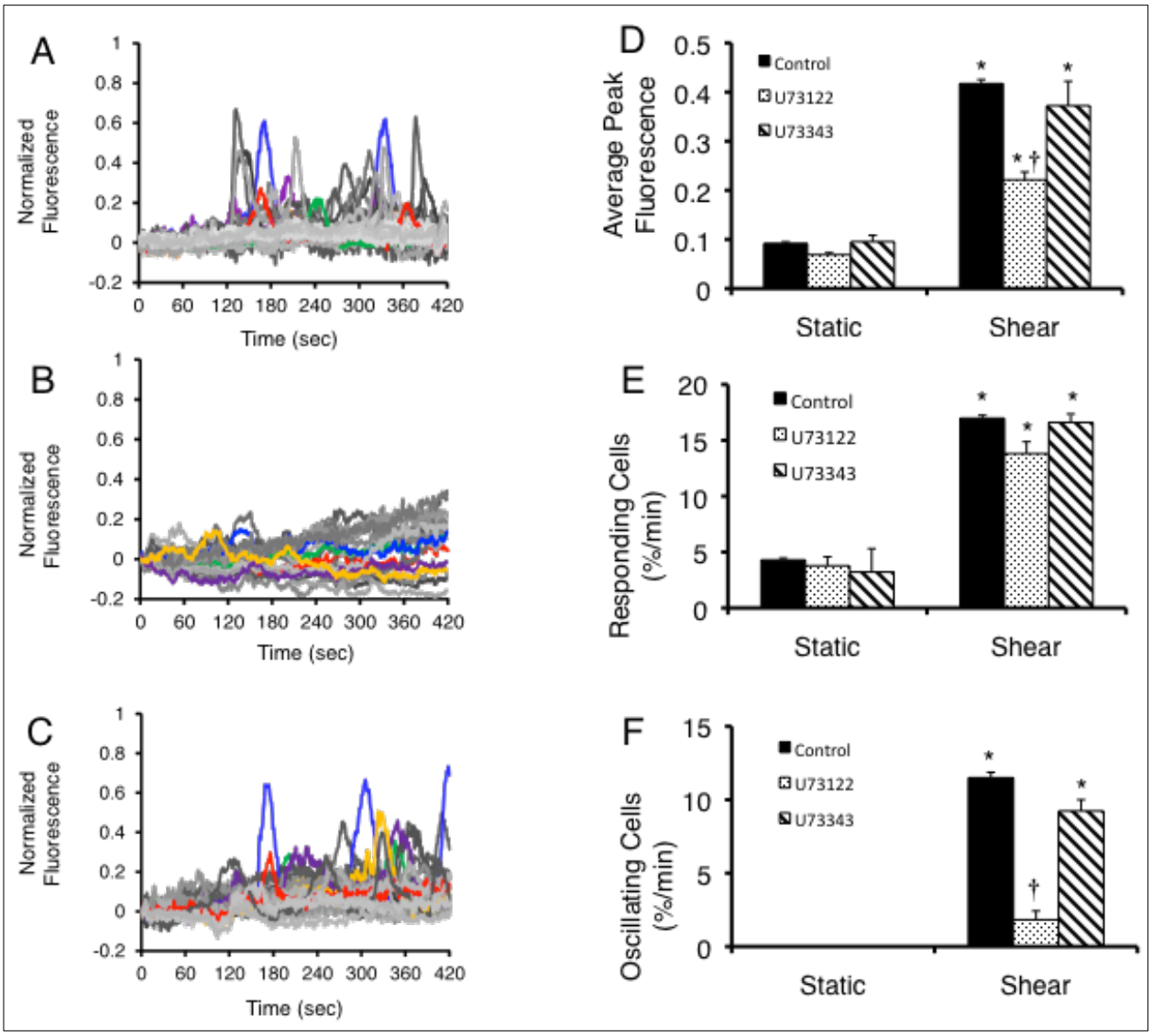

Figure 5. Effect of intracellular Ca^{2+} released via the IP_3R pathway on the $[Ca^{2+}]_i$ response to shear stress in ECs. A) Cells exposed to shear (10 dynes/cm^2) showed oscillatory rises in $[Ca^{2+}]_i$. B) Inhibition of PLC with U71322 hindered the shear response. C) Treatment with U73343 caused minimal change D) Average peak response of responding cells only compared to control. E) % Oscillating cells (defined as peaking more than once) per min. F) % Responding cells (defined as having a significant peak) per min. * $P<0.05$ compared to static of same chemical treatment. † $P<0.05$ compared to control treatment of same mechanical treatment.

The % oscillating cells were effectively unchanged between static and shear, meaning the PLC/IP_3 pathway to ER Ca^{2+} release plays a large role in maintaining the oscillations of $[Ca^{2+}]_i$ in response to shear. On the other hand, the average peak fluorescence was reduced, but still

significantly different to the static period, possibly due to insufficient PLC inhibition or another intracellular pathway being partly responsible to the response. The role of PLC/IP_3 pathway was confirmed when the inactive analog produced no differences in the response compared to control. Another interesting observation was that the IP_3 pathway did not have an effect on the actual ability of cells to respond, in terms of % responding/min. One theory is that the U73122 did not fully inhibit all IP_3 production by the PLC, leading to small amounts of ER Ca^{2+} release. All in all, it is clear the PLC/IP_3 pathway plays a major role in both the initial responses, and maintenance of oscillations, but may not be the sole factor.

Chapter III. Effects of Mitochondria on Calcium Response

The mitochondria maintain an electrochemical gradient by pumping positive hydrogen ions out of its membrane in the process of creating ATP. This gradient is then utilized in many pathways; most notably, it creates a drive for positive ions such as Ca^{2+} to enter[4,12,19,21]. The mitochondria are the second greatest store of intracellular Ca^{2+} and have been shown to uptake Ca^{2+} in response to chemical stimuli, most likely through the mitochondrial Ca^{2+} uniporter (MCU). To determine whether or not mitochondria also play a part in the response to shear stress, ECs were subjected to four different treatments. The first two were treatments with either 0.5 μM or 2 μM of p-trifluoromethoxyphenylhydrazone (FCCP), an agent that uncouples the mitochondria and dissipates the mitochondrial membrane potential. FCCP should in turn render the mitochondria partially or fully incapable of uptaking Ca^{2+} by regular means (**Figure 6A and 6B**). The third treatment used 2 μM oligomycin, an inhibitor of the mitochondrial ATP synthase. This was to make sure the hindrance of mitochondrial production of ATP was not the reason for the shear response using FCCP (**Figure 6C).** The final treatment used 20 μM of CGP37157, an inhibitor of the mitochondrial sodium-calcium exchanger (mNCX). Apart from the mPTP during pathological conditions, the NCX is the mitochondria's main source of Ca^{2+} extrusion back to the cytosol. As before, the cells were preincubated in HBSS and connected to the flow system. Directly before connection, ECs were flushed with HBSS in the presence of the compound under study. Results can be seen in **Figure 6D**.

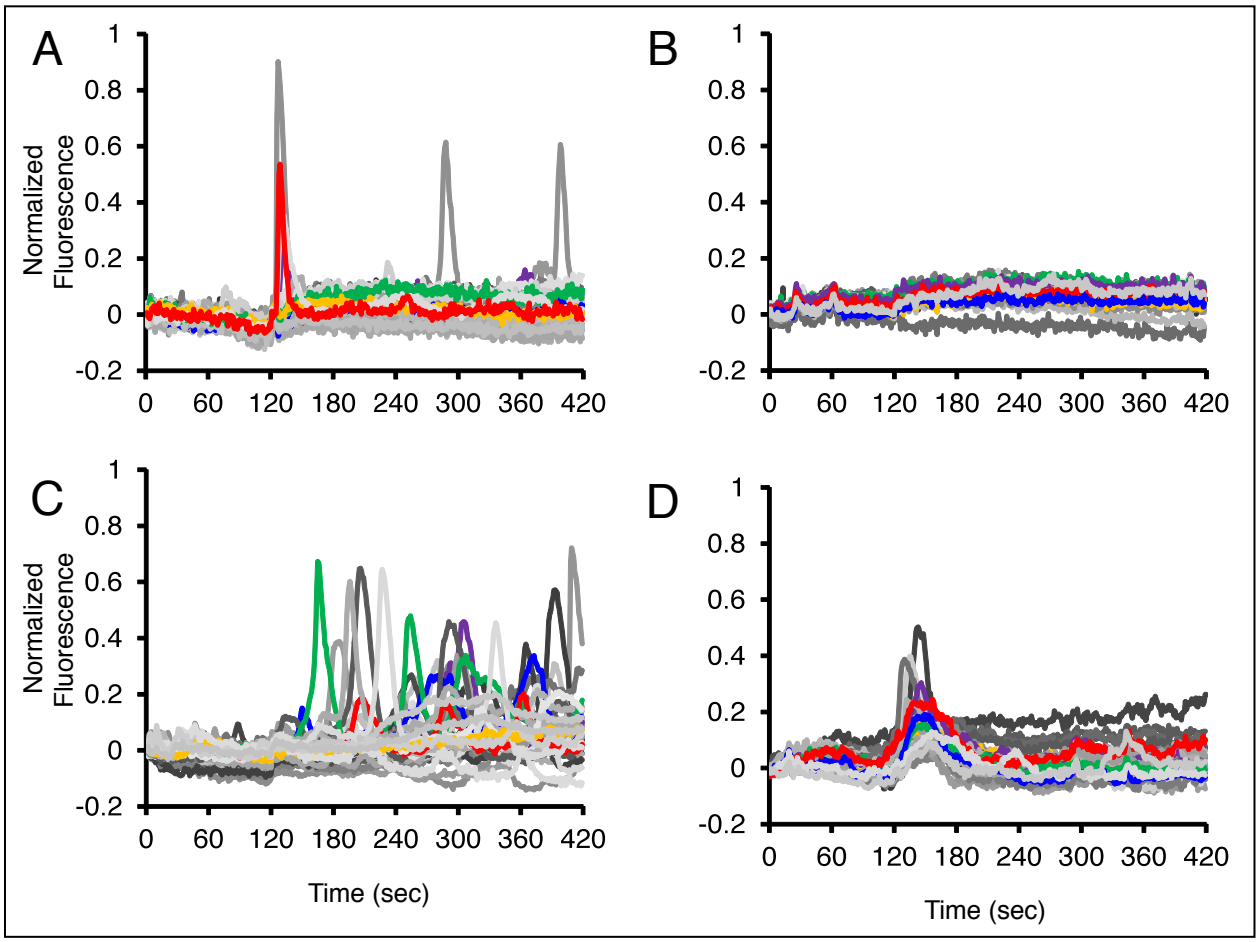

Figure 6. Effect of mitochondria on the response to shear stress in endothelial cells. A) 0.5 μM FCCP treatment, a mitochondrial uncoupler, partly blocking mitochondrial calcium uptake B) 2 μM FCCP treatment, fully blocking mitochondrial calcium uptake C) Oligomycin (2 μM), an ATP synthase inhibitor D) CGP37157 (20 μM), a mNCX inhibitor, blocking mitochondrial calcium efflux.

When the mitochondrial uptake was partially inhibited via 0.5 μM FCCP, or fully inhibited via 2 μM, the average peak values were significantly reduced or effectively eliminated, respectively. The % responding and % oscillating cells were affected similarly. Oligomycin had no significant effect as opposed to the control in any of the three parameters. CGP37157 did not have an effect on the % responding, but eliminated oscillations and significantly reduced the average peak fluorescence; however, it did not abolish them (**Figure 7**)

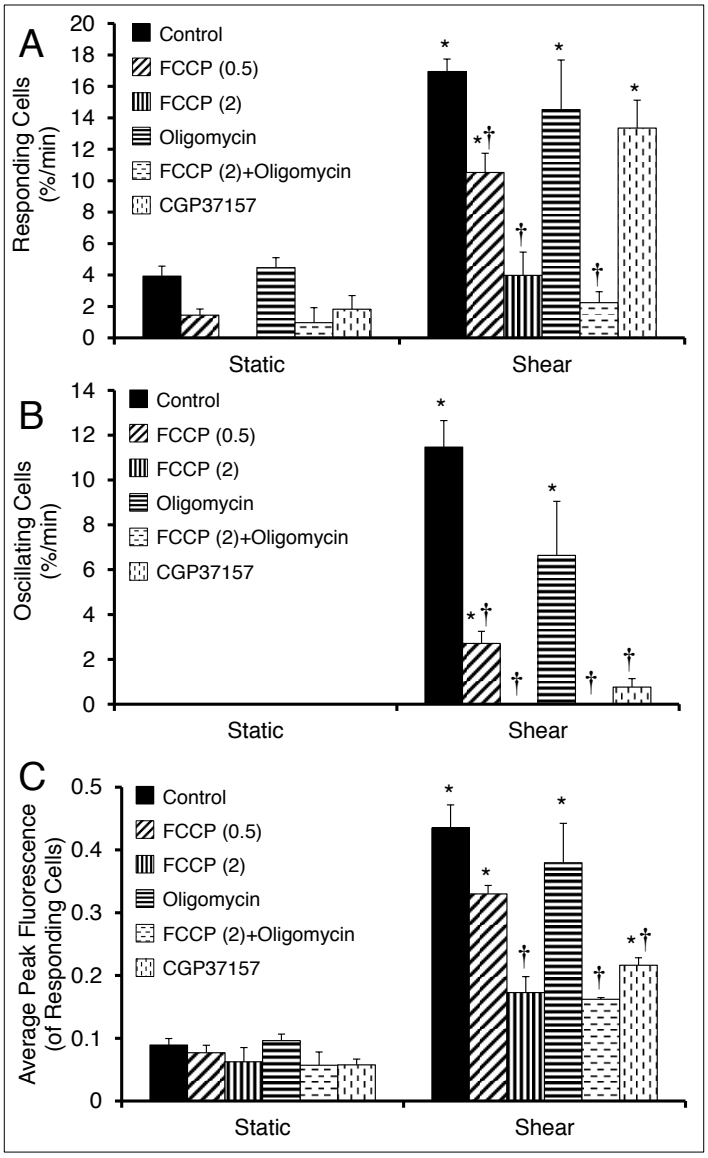

Figure 7. Effect of mitochondria on average peak fluorescence, % responding, and % oscillating ECs during shear experiments under different treatments A) % Responding cells per min B) % Oscillating cells per min C) Average peak fluorescence of only the responding cells
* $P<0.05$ compared to static of same chemical treatment. † $P<0.05$ compared to control treatment of same mechanical treatment.

Without the ability to buffer the $[Ca^{2+}]_i$, the response was diminished, and without the ability to release Ca^{2+}, the oscillations were eliminated. This could be due, in part, to the negative feedback at high levels of Ca^{2+} that the IP_3R experiences. Because of the close proximity of the mitochondria and IP_3R, a microdomain with a $[Ca^{2+}]$ higher than the rest of the cytosol forms. In

order to retain the ability to release more, Ca^{2+} must first be eliminated at this microdomain through the MCU. This Ca^{2+} could then be extruded through the mNCX. With this information, it seems that the ER and mitochondria exchange Ca^{2+} back and forth, thus causing the $[Ca^{2+}]_i$ oscillations.

Chapter IV: Conclusion

Understanding the pathway by which ECs respond to shear stress caused by flow in the vascular system could pave the way for many more experiments investigating the health of human blood vessels. Through this work, we were able to determine that the Ca^{2+} response to shear stress is mostly a result of Ca^{2+} release through the ER IP_3R channels. We were also able to determine that the mitochondria play a vital role in this process. With future work, we would like to delineate the process by which mitochondria are able to uptake the Ca^{2+}, specifically if it is indeed through the recently cloned MCU. This would be done through an MCU inhibitor Ruthenium Red or by using ECs isolated from MCU knockout mice. Another goal is to develop a mathematical model that can simulate known responses to shear stress experiments, with the goal being to predict the effects of different fluid mechanical forces on $[Ca^{2+}]_i$. It would also be beneficial to determine the exact route by which the ECs detect the mechanical stresses. With all this knowledge, future studies could begin looking at the complex interplay among Ca^{2+} and other factors such as NO and ROS.

With the results obtained in this work, we hypothesize that when ECs initially experience shear stress, the IP_3 pathway is activated, possibly through the binding of released ATP to P2Y receptors. IP_3 then binds to the IP_3R on the ER, eliciting a release of Ca^{2+}, which is taken in by the MCU at the ER-mitochondrial microdomains. This allows more Ca^{2+} to be release because the IP_3R is no longer hindered by high Ca^{2+} levels. Meanwhile, Ca^{2+} is slowly extruded through the mNCX into the cytosol, where the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) takes

it in to refill the ER. Our proposed pathway of endothelial Ca^{2+} homeostasis is schematically depicted in **Figure 8**.

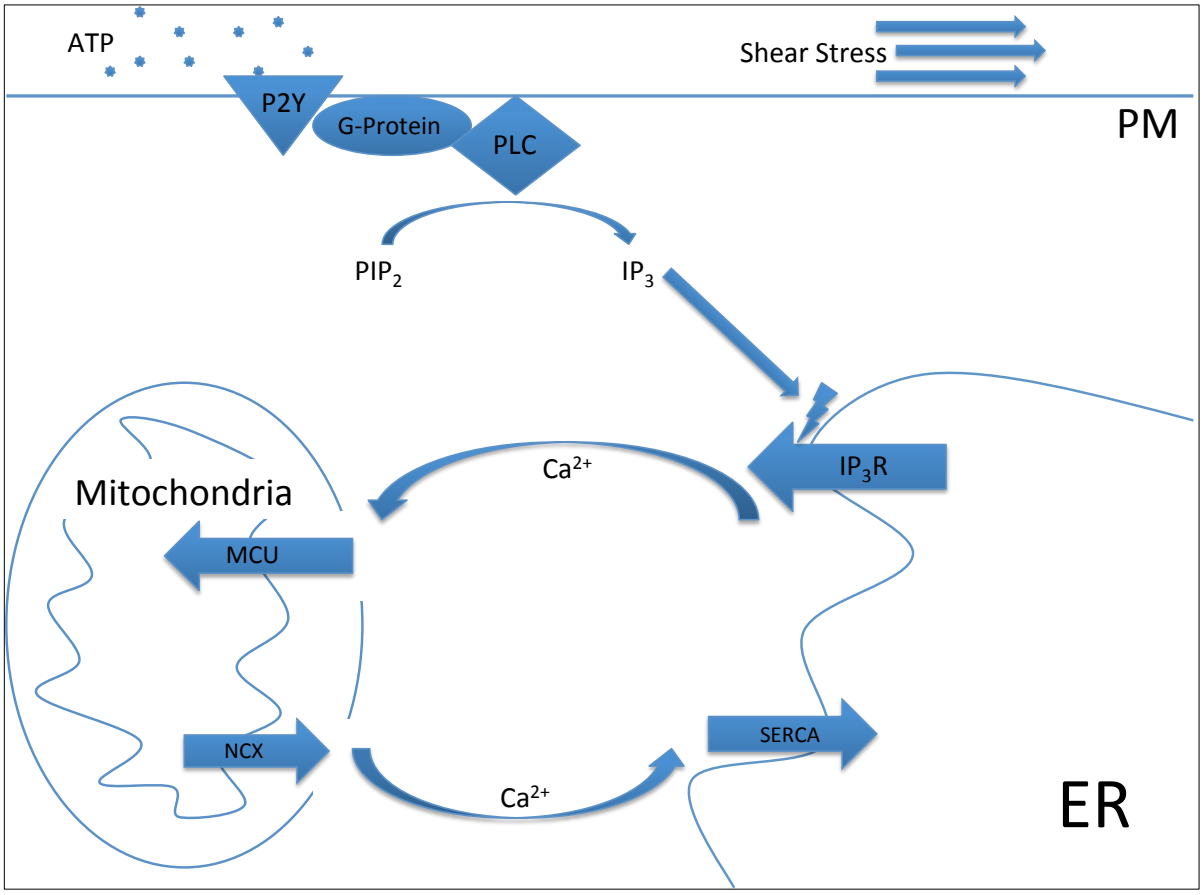

Figure 8. Schematic EC $[Ca^{2+}]_i$ response to shear stress. The IP_3 pathway is activated, which causes the IP_3R to release Ca^{2+} into the ER-mitochondria microdomain. This is buffered by the mitochondria through the MCU, and then extruded slowly through the mNCX. The ER slowly fills through the SERCA. We have not determined the process of sensing mechanical stimuli, but P2Y proteins on the PM are the most likely candidate.

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